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Title:

The effect of leukotrienes B and selected HETEs on the proliferation of colon cancer cells

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Abstract (English):

Eicosanoids have been implicated in colon carcinogenesis, but very little is known on the potential role of **leukotrienes** (LTs) and hydroxyeicosatetraenoic acids (HETEs) in this process; such compounds are produced by colonocytes and tumor infiltrating leukocytes. We studied the effect of LTB₄, LTB₄ methyl ester, LTB₅, 12(R)-HETE, 12(S)-HETE and 15(S)-HETE (10⁻¹⁰, 10⁻⁸, 10⁻⁶ M) on the proliferation rate, the cell cycle distribution, and the rate of apoptosis in HT-29 and HCT-15 human colon carcinoma cells. Our data show that LTB₄, a lipoxygenase product, increased the proliferation rate of both cell lines in a time- and concentration-dependent manner. In HT-29 cells the concentration-response curve was bell-shaped (maximal effect at 10⁻⁸ M). The proliferative effects of LTB₄ in HT-29 cells were inhibited by SC-41930, a competitive antagonist of LTB₄, suggesting the existence of an LTB₄ receptor in epithelial cells. The methyl ester of LTB₄ stimulated the proliferation of these cells, but LTB₅, an isomer of LTB₄ derived from eicosapentaenoic acid, did not. Of the HETEs, only 12(R)-HETE, a P-450 product, stimulated the proliferation of both cell lines; the other HETEs, all lipoxygenase products, failed to affect the proliferation of these cells. None of these eicosanoids had any effect on cell cycle distribution or apoptosis in either cell line. Taken together with our previous data showing that PGs stimulate colon cancer

cell proliferation (Qiao et al. (1995) Biochim. Biophys. Acta 1258, 215-223), these findings indicate that arachidonic acid products synthesized via at least three different pathways (cyclooxygenase, lipoxygenase, *P*-450) may be able to modulate the growth of colon **cancer**, and suggest a potential role in human colon carcinogenesis for LTB₄ and 12(*R*)-HETE.

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TI Effect of the specific cyclooxygenase-2
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AU Hussey H J; Tisdale M J
CS Pharmaceutical Sciences Research Institute, Aston University, UK.
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AN 1999:441597 BIOSIS
DN PREV199900441597
TI Expression of IL-1beta, IL-6, TNF-alpha and Cox-2 in the brain
of tumor bearing mice with systemic inflammation and progressive anorexia-
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AU Wang, Wenhua (1); Lundholm, Kent (1); Svanberg, Elisabeth (1)
CS (1) Dept of Surgery, Sahlgrenska University Hospital, Goteborg Sweden
SO Journal of Interferon and Cytokine Research, (Sept., 1999) Vol. 19, No.
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AU Cahlin, Christian; Gelin, Johan; Delbro, Dick; Lonnroth, Christina; Doi,
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Effect of Cyclooxygenase and Nitric Oxide Synthase Inhibitors on Tumor Growth in Mouse Tumor Models with and without Cancer Cachexia Related to Prostanoids¹

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ABSTRACT

The potential interaction between cyclooxygenase (Cox) and NO metabolic pathways in the control of local tumor growth was evaluated. Mice bearing either a sarcoma-derived tumor (C57Bl; MCG 101) or a malignant melanoma (C3H/HeN; K1735-M2) were used. These models were principally different because they demonstrate, in tumor hosts, conditions with and without cancer cachexia, seemingly related to high and low production of prostanoids, respectively. Cox inhibitors (Cox-1 and Cox-2) decreased tumor growth by 35–40% in MCG 101-bearing mice but had no such effect on melanoma-bearing mice, despite the expression of the Cox-2 protein in melanoma cells. Indomethacin reduced prostanoid production in both tumor (MCG 101) and host tissues and reduced tumor cell proliferation, mainly *in vivo*. Nitric oxide synthase (NOS) inhibitors (*N*^ω-nitro-L-arginine methyl ester and *N*^ω-nitro-D-arginine) reduced tumor growth *in vivo* by ~50% in both tumor models. Tumor growth reduction, related to NOS inhibition, was unrelated to prostanoid production and was an *in vivo* phenomenon in both tumor models. Specific inhibitors of inducible NOS activity, unexpectedly, had no effect in any tumor model, although inducible NOS protein was present in tumor tissues in large amounts. A combination of Cox and NOS inhibitors had no additive effect on tumor growth (MCG 101). Cox inhibition increased tumor tissue (MCG 101) expression of cNOS mRNA but had no significant effect on tumor tissue expression of the transferrin receptor, vascular endothelial growth factor, or basic fibroblast growth factor. NOS inhibition increased tumor tissue content of cNOS mRNA but showed as well a trend to increase mRNA content of the transferrin receptor and vascular endothelial growth factor. Our results suggest that NOS inhibitors can decrease the local growth of tumors that are either responsive or unresponsive to Cox inhibition. This effect may reflect cross-talk between Cox and NOS pathways within or among tumor cells, or it may represent unrelated effects on tumor and host cells. Whether NO inhibition may be used therapeutically in clinical tumors that are unresponsive to eicosanoid intervention remains to be evaluated.

INTRODUCTION

Tumor growth with subsequent cancer cachexia is mediated by complex interactions of cytokines and growth factors, partly communicated by eicosanoids (1). Accordingly, it has been reported that a variety of Cox³ inhibitors attenuate experimental tumor growth (2), tissue invasion (3), and influence on carcinogenesis (4) and prolong survival in experimental animals (5), and probably in clinical cancer (6) as well. Thus, indomethacin, a classic prostaglandin synthase

inhibitor, attenuated cancer cachexia, counteracted tumor-related anorexia, and decreased tumor growth in order of magnitudes observed following provision of neutralizing antibodies to cytokines such as IL-1 α , IL-1 β (7), tumor necrosis factor- α (8), and IL-6.⁴ Thus, the improvement of cachexia and depression of tumor growth by Cox inhibition (Cox-1 and Cox-2) may be related to reduced production of prostanoids in either tumor cells or host tissues, or a combination of both.

NOS activities are also related to tumor growth, cellular differentiation, tumor blood flow, and metastatic formation under similar conditions in which Cox inhibitors may act (9–13). Interestingly, it has been recognized that production of NO is related to Cox metabolism both *in vitro* and *in vivo* during a variety of experimental conditions (14–16). Therefore, it is possible that both NO and Cox activities are either alternative or common pathways in the regulation of fundamental processes that determine local and systemic progression of cancer (17). The aim of the present study was, therefore, to evaluate to what extent Cox and NO-forming pathways may interact in their influence on tumor growth and subsequent development of cancer cachexia. For this purpose, we have used two different mouse tumor models with and without cancer cachexia related to high and low prostanoid production, respectively.

MATERIALS AND METHODS

Tumor Models

The present *in vivo* study of tumor growth was undertaken in weight-stable (20–24 g) female mice C57Bl, implanted with MCG 101 tumors, and in C3H/HeN implanted with K1735-M2 melanoma cells (a kind gift from Prof. I. Fidler, The University of Texas M. D. Anderson Cancer Center, Houston, TX). MCG 101 is a nonmetastasizing, undifferentiated epithelial-like solid tumor. This tumor model has been used extensively in our laboratory for the study of cancer cachexia, and it has been grown continuously *in vivo* since 1972 (18, 19). When implanted s.c., the tumors grow locally with a reproducible growth pattern. The animals die because of cancer cachexia 12–15 days after tumor implantation. The MCG tumor produce PGE₂, both *in vitro* and *in vivo*, leading to elevated plasma concentrations of PGE₂ (1). Cox inhibition by indomethacin, which normalizes systemic levels of PGE₂, improves nutritional state and food intake, reduces tumor growth, and prolongs survival (20). In contrast, the K1735-M2 melanoma synthesizes considerably less PGE₂ and did not produce overt cachexia during tumor progression. Thus, melanoma-bearing mice with extensive local tumor growth did not die with cachexia. Pulmonary metastases are probably the cause of death beyond 4–5 weeks after tumor implantation (21). Consequently, indomethacin treatment in this Cox-unresponsive model had no influence on either tumor growth, nutritional state, or survival.

All animals were housed in groups of five in plastic cages in a temperature-controlled room (24°C) at increased humidity with a 12-h light/dark cycle and were provided free access to standard chow. Tumor tissue or cultured cell suspensions were inoculated under light i.p. anesthesia (100 μ g/kg ketamin and 5 μ g/kg xylazin). C57Bl mice were implanted s.c. with 3 mm³ of MCG tumor tissue on both sides of the back to allow unrestrained movement of the animals. The K1735-M2 cell line was suspended in McCoy's 5A medium in a concentration of 500,000 cells/ml, of which 0.2 ml containing 100,000 cells was inoculated bilaterally in the flanks of the C3H/HeN animals.

Mice were killed by cervical dislocation either 10 days (MCG) or 30 days

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³ The abbreviations used are: Cox, cyclooxygenase; IL, interleukin; NOS, nitric oxide synthase; cNOS, constitutive NOS; iNOS, inducible NOS; PGE₂, prostaglandin E₂; L-Name, *N*^ω-nitro-L-arginine methyl ester; D-Name, *N*^ω-nitro-D-arginine methyl ester; L-NoArg, *N*^ω-nitro-L-arginine; Amg, aminoguanidine hemisulfate; Smt, S-methylisothiourea sulfate; L-745,337, 5-methane sulfonamido-6-(2,4-difluoro-thiophenyl)-1-indanone; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor.

⁴ Unpublished observations.

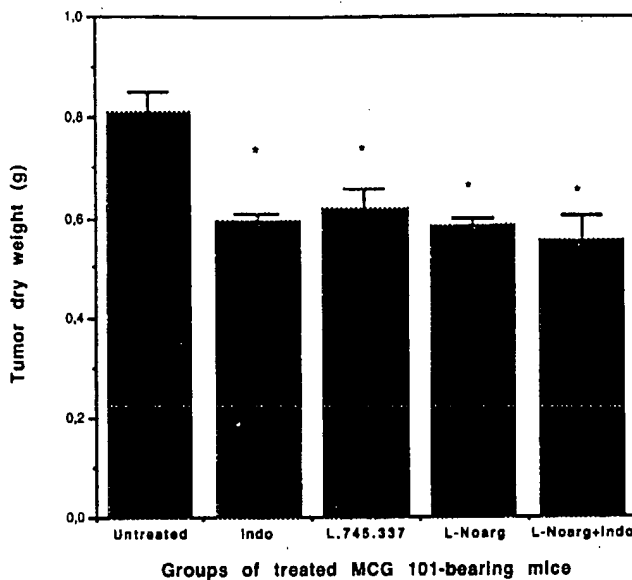


Fig. 1. The effect of Cox [indomethacin, Cox-1, and Cox-2]; (L-745,337 and Cox-2)) and NOS inhibition (L-NoArg and NOS) on tumor growth in MCG 101-bearing mice 10 days after tumor inoculation. The drugs were provided in the drinking water as described in "Materials and Methods." At least 10 animals were used in each group. *, $P < 0.01$ compared with untreated mice. Bars, SE.

(K1735-M2) after tumor implantation. The tumors were dissected free for compositional measurements. Food intake, body composition, and tumor dry weight were measured as described elsewhere (22, 23).

Provision of Drugs

Most drugs were provided in the drinking water to minimize the stress reaction by daily injections. Calculation of the dosage of each drug administered was based on measured water consumption corresponding to ~3 ml/animal and 24 h (20). Otherwise, a Cox-2 inhibitor (L-745,337) was given as s.c. daily injections in saline, according to recommendation of the provider (24). Two hundred and fifty mice were randomly divided into 16 groups, which received the following treatments with standardized doses.

Experiments in MCG-bearing Mice

For NOS inhibition, the groups were divided as follows: group 1, L-Name (100 mg/kg/day; $n = 17$); group 2, L-NoArg (100 mg/kg/day; $n = 12$); group 3, L-Name plus L-NoArg (100 mg/kg/day of each; $n = 9$); and group 4, D-Name (100 mg/kg/day; $n = 6$). For iNOS inhibition, the groups were divided as follows: group 5, Amg (100 mg/kg/day; $n = 17$); and group 6, Smt (50 mg/kg/day; $n = 8$). For Cox inhibition, there was only one group: group 7, indomethacin sodium, (Confortid; 1 mg/kg/day; $n = 22$), which was regarded as an unselective Cox-1/Cox-2 inhibitor. For Cox-2 inhibition, there was only one group: group 8, L-745,337 (10 mg/kg/day; $n = 26$). For the combination of Cox and NOS inhibitors, the groups were divided as follows: group 9, indomethacin combined with L-Name (1 mg/kg/day plus 100 mg/kg/day; $n = 7$); and group 10, indomethacin combined with L-NoArg (1 mg/kg/day

plus 100 mg/kg/day; $n = 12$). There was one control group: group 11 ($n = 54$). These tumor-bearing mice received normal drinking water only.

Experiments in K1735-M2-bearing Mice

For NOS inhibition, there was only one group: group 12, L-NoArg ($n = 10$). For iNOS inhibition, the groups were divided as follows: group 13, Amg ($n = 10$); and group 14, Smt ($n = 10$). For Cox inhibition, there was only one group: group 15, indomethacin ($n = 10$). There was one control group: group 16 ($n = 20$). These tumor-bearing mice received normal drinking water only.

L-Name, D-Name, Amg, and L-NoArg substances were from Sigma Chemical Co. (St. Louis, MO). Smt was from Fluka Chemika (Buchs, Switzerland). Indomethacin (Confortid) was from Dumex A/S (Copenhagen, Denmark), provided at effective doses as confirmed by Gelin *et al.* (20). L-745,337 was a kind gift from Merck Frosst, Canada Inc. The suggestions of effective *in vivo* doses of NOS inhibitors were based on published reports (25, 26).

Cell Cultures

Tumor cells were grown and harvested for analyses as described earlier (1).

Immune Histochemistry

Tumors were removed and fixed in 4% paraformaldehyde in PBS for 3 days, rinsed, and embedded in paraffin. Tissue sections of 3–4 μ m were cut. After deparaffination, rehydration, and rinsing, sections were trypsinated for 15 min at 37°C in 0.04% trypsin (Sigma T-8253), 0.01% CaCl_2 in Tris-buffered saline (TBS). After blocking of endogenous biotin activity (Avidin/Biotin blocking kit; Vector), sections were incubated for 30 min in 5% lipid-free dry milk in TBS. For NOS detection, (endothelial) sections were incubated 30 min in normal goat IgG (Santa Cruz Biotechnology) and diluted 1:500 in dry milk/TBS, and Cox sections were incubated 30 min in normal rabbit IgG (Santa Cruz Biotechnology) diluted 1:500. Test sections were incubated with antibodies against cNOS (endothelial isoform) diluted 1:200, iNOS diluted 1:200 (Santa Cruz Biotechnology), Cox-1 diluted 1:200, and Cox-2 diluted 1:200 (Santa Cruz) overnight at 4°C. Control sections (NOS) were incubated in normal rabbit IgG, diluted 1:500, and control sections (Cox) were incubated in normal goat IgG, diluted 1:500 or with neutralized antibodies overnight. For neutralization, antibodies were reacted with a 10-fold (by weight) excess of peptide antigens in 5% dry milk in TBS, 2 h at 37°C. Cox-1 peptide and Cox-2 peptide were from Santa Cruz Biotechnology. Sections were exposed 30 min at room temperature to biotinylated second antibodies: NOS, antirabbit diluted 1:200 (Santa Cruz Biotechnology); Cox, antigoat diluted 1:500 (Dakopatts), followed by 30 min incubation in alkaline phosphate-conjugated streptavidin diluted 1:150 (Amersham). Fast Red (Dakopatts) was used as substrate, and counterstaining was done in Mayers hematoxylin. Mount-Quick Aqueous (Daido Sangyo Co, Ltd, Japan) was used for mounting.

Western Blot

Tumor tissue was rinsed from debris in 4°C normal saline and was placed in plastic test tubes and snap frozen in liquid nitrogen. Specimens were thereafter stored at -84°C until analyses. Tissues were homogenized by a glass pestle in 4 volumes of PE buffer (10 mM potassium phosphate buffer, pH 6.8, and 1 mM EDTA) containing 10 mM 3-[(3-cholamidopropyl)dimethylammonio]1-propanesulfonate (Sigma Chemical Co.), 0.15 μ M aprotinin, 2.0 μ M leupeptin, 4.0 μ M Pefabloc, and 15 μ M pepstatin (all from Boehringer Mannheim, Mannheim, Germany; Ref. 27). The homogenate was centrifuged

Table 1 Body composition in MCG 101^a-bearing mice treated daily with indomethacin 1.0 μ g/g body weight or NoArg 200 μ g/g body weight as described in "Materials and Methods"

	<i>n</i>	Initial body weight (g)	Final body weight including tumor (g)	Cumulative food intake (g)	Carcass dry weight (g)	Carcass fat-free weight (g)	Body fat (g)	Tumor wet weight (g)	Tumor dry weight (g)
TB ^b -indo	10	18.7 \pm 0.2	21.5 \pm 0.5	25.8	5.835 \pm 0.1	4.264 \pm 0.06	1.773 \pm 0.08	2.048 \pm 0.2	0.364 \pm 0.03
TB-control	8	18.5 \pm 0.1	21.7 \pm 0.5	20.1	5.557 \pm 0.2	4.079 \pm 0.07	1.717 \pm 0.1	3.054 \pm 0.3	0.545 \pm 0.05
<i>P</i>		NS	NS	<0.05 ^c	NS	<0.03	NS	<0.006	<0.004
TB-NoArg	12	21.2 \pm 0.4	21.6 \pm 0.3	26.1	6.458 \pm 0.2	4.633 \pm 0.1	1.732 \pm 0.2	1.021 \pm 0.1	0.172 \pm 0.02
TB-control	12	21.1 \pm 0.5	21.7 \pm 0.5	26.4	6.284 \pm 0.2	4.365 \pm 0.1	1.787 \pm 0.1	1.792 \pm 0.2	0.311 \pm 0.04
<i>P</i>		NS	NS	NS	NS	<0.03	NS	<0.002	<0.002

^a Data on carcass composition and tumor growth in melanoma-bearing mice are not given because these mice were nutritionally unaffected by the melanoma.

^b TB, tumor-bearing.

^c Calculated based on standard variations reported in a previous publication (20).

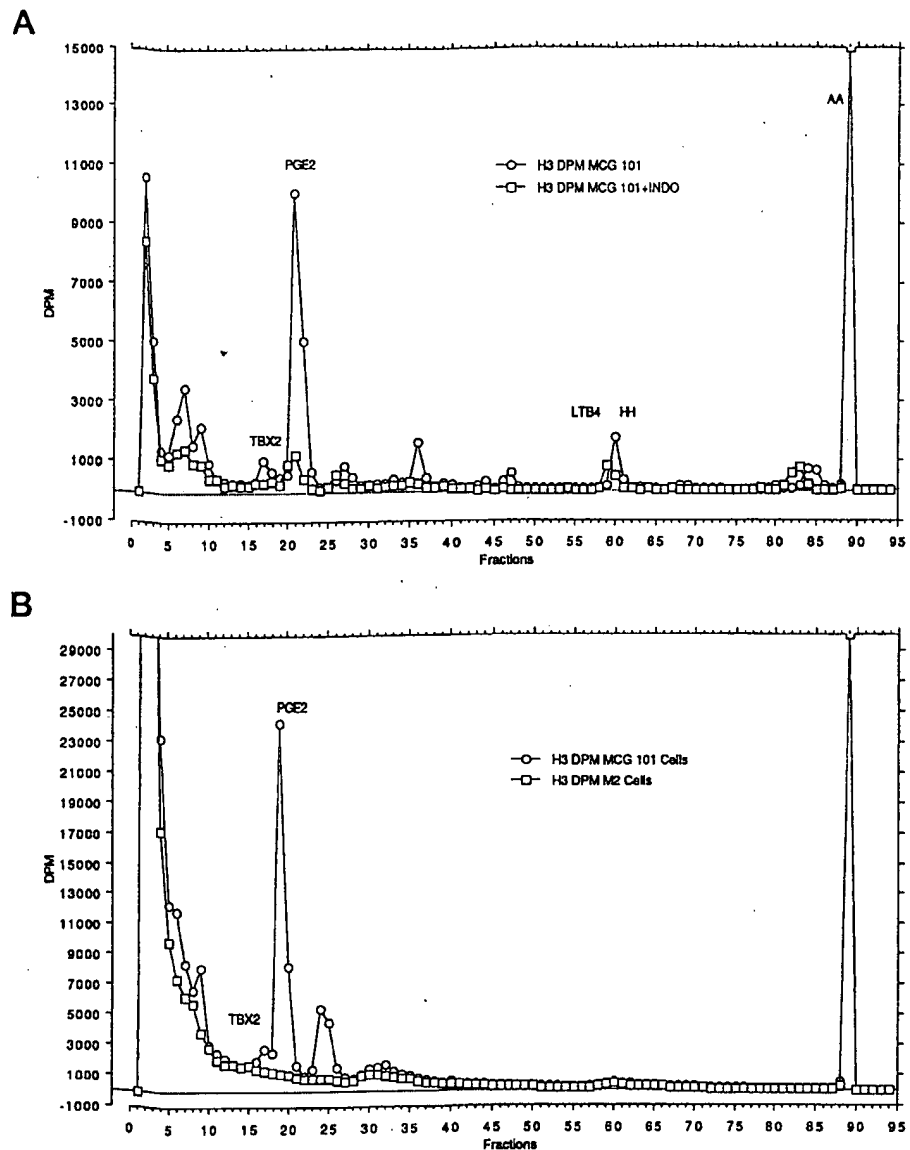


Fig. 2. A, the chromatogram shows a representative profile of eicosanoids synthesized from tritiated arachidonic acid in tissue homogenates prepared from MCG 101 tumors from mice without (○) and with □ indomethacin treatment before sacrifice, as described in "Materials and Methods." B, eicosanoids synthesized in cultured MCG 101 cells compared with the synthesis in K1735-M2 cells. For details, see "Materials and Methods." Eicosanoids were separated as described (32). Identified peaks were thromboxane B2 (TBX2), PGE₂, leukotriene B4 (LTB₄), hydroxy-heptadecatrienoic acid (HH), and arachidonic acid (AA).

(10,000 × g for 10 min) after determination of protein concentration in the supernatant (28). The samples were stored at -84°C until analysis. Total protein-equivalents (50 mg/each sample) were diluted in SDS sample buffer and were separated on 7.5% SDS polyacrylamide gel (Bio-Rad Laboratories, Solna, Sweden) in the Laemmli buffer system (29). Proteins were transferred to a polyvinylidene difluoride membrane (Amersham Laboratories, Amersham, Buckinghamshire, United Kingdom), which was incubated with polyclonal rabbit anti-iNOS, or monoclonal mouse anti-eNOS antibody (diluted 1:1,500 and 1:250, respectively) overnight at 4°C. The antibodies were purchased from Transduction Laboratories (Lexington, KY; anti-iNOS and anti-eNOS). Bands were detected by chemiluminescence using an alkaline phosphatase-conjugated secondary antibody and CSPD (Western-Light; Tropix, Inc., Bedford, MA; Ref. 30). Membranes were exposed to ECL film (Amersham Laboratories).

Tumor Tissue Expression of NOS and Growth Factors

Specimens of viable tumor tissue from a pool of five mice in each group were used for extraction of total cellular RNA, using guanidinium isothiocyanate, according to standard procedure (31). Poly(A)⁺ RNA was selected, and 5–8 µg were either glyoxal treated and separated in 1.2% agarose gels or run in gels containing formaldehyde and capillary transferred to Hybond N⁺

membranes (Amersham). Murine DNA probes were made by PCR technique using a cDNA library, reversed transcribed from mRNAs from liver or WBCs. Primers were chosen from Mac Vectors database Entrez to give fragments of about 350 bp in length when amplified. These probes were labeled with [α -³²P]dCTP in a random priming reaction using an oligolabeling kit (Roche Diagnostics Scandinavia or Pharmacia-Biotech). Oligonucleotides of 30–40 bp were also used as probes. A mix of four murine antisense oligonucleotides/gene were used to probe VEGF, bFGF, angiogenin, the transferrin receptor, cNOS, and β -actin. These were 3'-end labeled using [α -³²P]ddATP (Amersham). Prehybridization and hybridization were performed for 24 h each at 42°C (in a Robbins oven) in 50% formamide, 5× SSPE, 0.5% SDS, 10× Denhardt's solution, and 250 µg/ml denatured low molecular weight DNA, and 50 µg/ml polyadenylic acid. Probe concentration was 5–10 × 10⁵ dpm/ml of hybridization solution. Postwashes were made in 5× SSPE/0.1% SDS with dilution to 0.1× SSPE/0.1% SDS, five concentrations in total for 30 min each at 42°C. Filters were exposed to Hyperfilm MP (Amersham) for 1–11 days. Densitometric analyses of autoradiograms were performed at 550 nm using a Shimadzu CS-930 densitometer.

Tumor tissue expression of Cox-2 and β -actin was measured by semiquantitative PCR. Sixty ng of poly(A)⁺ RNA from tumor tissue were reversed transcribed using GenAmp RNA PCR kit (Perkin-Elmer). Oligo d(T)₁₆ was

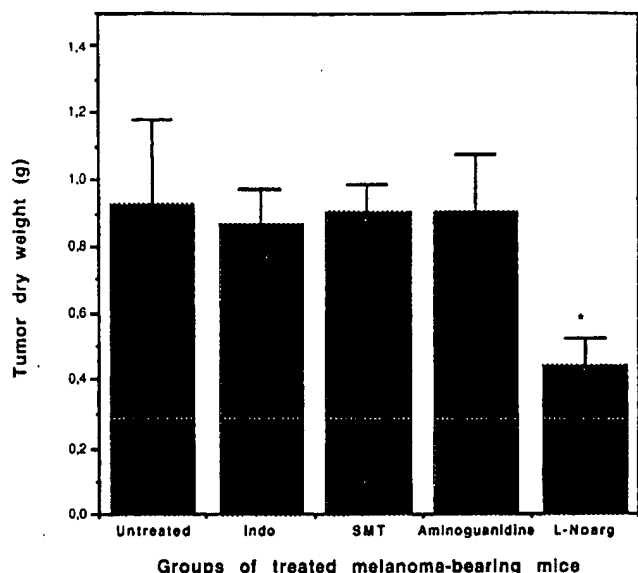


Fig. 3. The effects of inhibitors of inducible [Smt, aminoguanidine, (iNOS)] and constitutively expressed [L-NoArg, (NOS)] NOS on melanoma growth (K1735-M2) in C3H/HeN mice at 30 days after tumor implantation, compared with the effect of indomethacin. Drugs were provided in the drinking water as described in "Materials and Methods." At least 10 animals were used in each group. *, $P < 0.01$. Bars, SE.

used as a downstream primer. Amplification was done in a reaction volume of 50 μ l, using 1:20 volume of the reverse transcriptase-reaction, 2 mM $MgCl_2$, 200 μ M deoxynucleotide triphosphates, 0.15 μ M of each primer, and 1.25 units of AmpliTaq. HotStart was performed in HotStart 50 tubes (Molecular Bio-Products, Inc., San Diego, CA). Reaction cycles were: denaturing at 94°C for 1 min, annealing at 58°C for 1.2 min, and extension at 72°C for 1 min for 20–36 cycles, followed by a 5-min extension time at 72°C. Aliquots of the PCR reaction (10 μ l) were separated in 4% agarose gels and stained in ethidium bromide.

Prostaglandin E_2 (^{125}I) Assay System

Blood was collected from tumor-bearing animals on day 10 (MCG) and day 30 (melanoma) after inoculation. Healthy animals were used as normal controls. Sodium citrate was used as an anticoagulant, and indomethacin (10 μ g/ml) was used to prevent further breakdown of arachidonic acid. Specimens were kept on ice and centrifuged at $2500 \times g$ for 10 min at 4°C. After acidification, ethanol addition, and centrifugation of plasma, PGE_2 was extracted on Amprep C 18 minicolumns (Amersham RPN 1900), according to the recommendations in the PGE_2 assay system (Amersham RPA 530).

Tumor tissue was homogenized in 0.1 M Tris-HCl buffer (pH 7.4) containing indomethacin as inhibitor. All tubes were kept on ice. After centrifugation, supernatants were treated as mentioned above and applied to C_{18} minicolumns for extraction of PGE_2 . In conditioned media from tumor cell cultures, PGE_2 was extracted by the same procedure. After conversion of extracted PGE_2 by methyl oximation according to the kit instructions, RIA was performed within 6 days. Values were calculated from duplicates.

Fractionation of Eicosanoids

Tissue Preparation. Tumor tissue from five animals was placed in RPMI 1640 cell culture medium. A 10% homogenate was prepared in a glass tissue grinder, and 2 ml of the homogenate were incubated with [3H]arachidonic acid (Amersham; 2 μ Ci/ml homogenate) for 3 h at 37°C. Samples were then precipitated with 4 volumes of methanol, vortexed, and refrigerated for 20 min at 4°C. The samples were centrifuged at $1800 \times g$. The supernatant was collected and evaporated under N_2 . Finally, the samples were diluted with methanol to a final concentration of 30% methanol.

Cell Preparation. Tumor cells were grown in cell culture flasks and harvested 4 days after splitting. A cell suspension containing approximately 3.4×10^7 cells/ml was made. The cell suspension was homogenized in a

glass homogenizer, diluted with 1 volume of McCoy cell culture medium, and incubated with [3H]arachidonic acid (2 μ Ci/ml homogenate) for 3 h at 37°C. The samples were thereafter treated in the same way as tissue samples.

Tissue and cell extracts (100 μ l) were separated on a C_{18} , Pak column (Waters 5 μ m; 8-mm inside diameter radius). Eicosanoids were eluted during a series of isocratic elutions with mixtures of methanol and water buffered to pH 5.8, as described (32).

Statistics

Results are expressed as mean \pm SE. Multiple group comparisons were performed by one-way ANOVA. Scheffe F test was used post hoc. $P < 0.05$ was considered statistically significant.

The experimental protocol was approved by the Committee for Animal Ethics at Göteborg University.

RESULTS

Cyclooxygenase Inhibition and Eicosanoids. The present study confirms that both indomethacin (Cox-1 and Cox-2 inhibition) and more specific Cox-2 inhibition (L-745,337) decreased tumor growth by ~35–40% ($P < 0.01$; Fig. 1). Reduced tumor growth was related to attenuated anorexia and improved body composition in MCG-bearing mice (Table 1). Indomethacin had no such beneficial effects in melanoma K1735-M2-bearing mice (results not shown). Thus, melanoma-bearing mice remained nutritionally unaffected by melanoma growth throughout the entire experimental period (30 days), despite having a progressively larger tumor burden than MCG 101-bearing mice close to death. This discrepancy between the two tumor models may be linked to the fact that MCG 101 tumors showed a high production of prostanoids (mainly PGE_2 , $\approx 1000 \pm 100$ pg/ 10^6 cells/day), whereas the melanoma cells produced eicosanoids at low amounts ($\approx 10 \pm 5$ pg PGE_2 / 10^6 cells/day), evaluated both *in vivo* and in cultured tumor cells (Fig. 2).

High-performance liquid chromatography fractionation of eicosanoids, produced in either tumor tissue or in cultured tumor cells, confirmed that the main quantitative difference between MCG 101 and K1735-M2 cells was the production of PGE_2 . A manifold of arachidonic acid metabolites seemed to be present when eicosanoids in tissue specimens from *in vivo* growing tumors were fractionated compared with specimens from cultured tumor cells (Fig. 2). This difference may indicate that eicosanoids produced by host cells were also involved.

At the time of sacrifice, MCG 101-bearing mice had elevated plasma concentrations of PGE_2 in the range of 1015 ± 173 pg/ml, which decreased to 90 ± 15 pg/ml by indomethacin treatment and to 234 ± 39 pg/ml by L-744,337. Freely fed non-tumor-bearing control mice had plasma levels of 81 ± 24 pg/ml. The PGE_2 concentrations in tumor tissue were $\approx 1000 \pm 150$ ng/g in MCG 101 tumors 10 days after tumor implantation in untreated mice. This concentration decreased <20 ng/g by indomethacin treatment, which was comparable with concentrations of PGE_2 in untreated malignant melanoma-bearing mice (10 ± 2 ng/g tumor tissue) having plasma concentration of PGE_2 (<50 pg/ml) close to concentrations in healthy non-tumor-bearing mice. Provision of a Cox-2 inhibitor (L-745,337) decreased PGE_2 concentrations in MCG 101 tumors from 840 ± 57 to 180 ± 60 ng/g ($P < 0.01$).

NOS Inhibition. Inhibitors (L-NoArg and L-Name) of NO production decreased tumor growth *in vivo* by ~50% in both MCG 101 (Fig. 1) and melanoma-bearing mice ($P < 0.01$; Fig. 3). L-NoArg and L-Name reduced local tumor growth similarly at concentrations used (100 mg/kg body weight). The stereoisomer D-Name had no effect on tumor growth, demonstrating NOS specificity by L-NoArg and L-Name. NOS inhibition of tumor growth in MCG 101-bearing mice

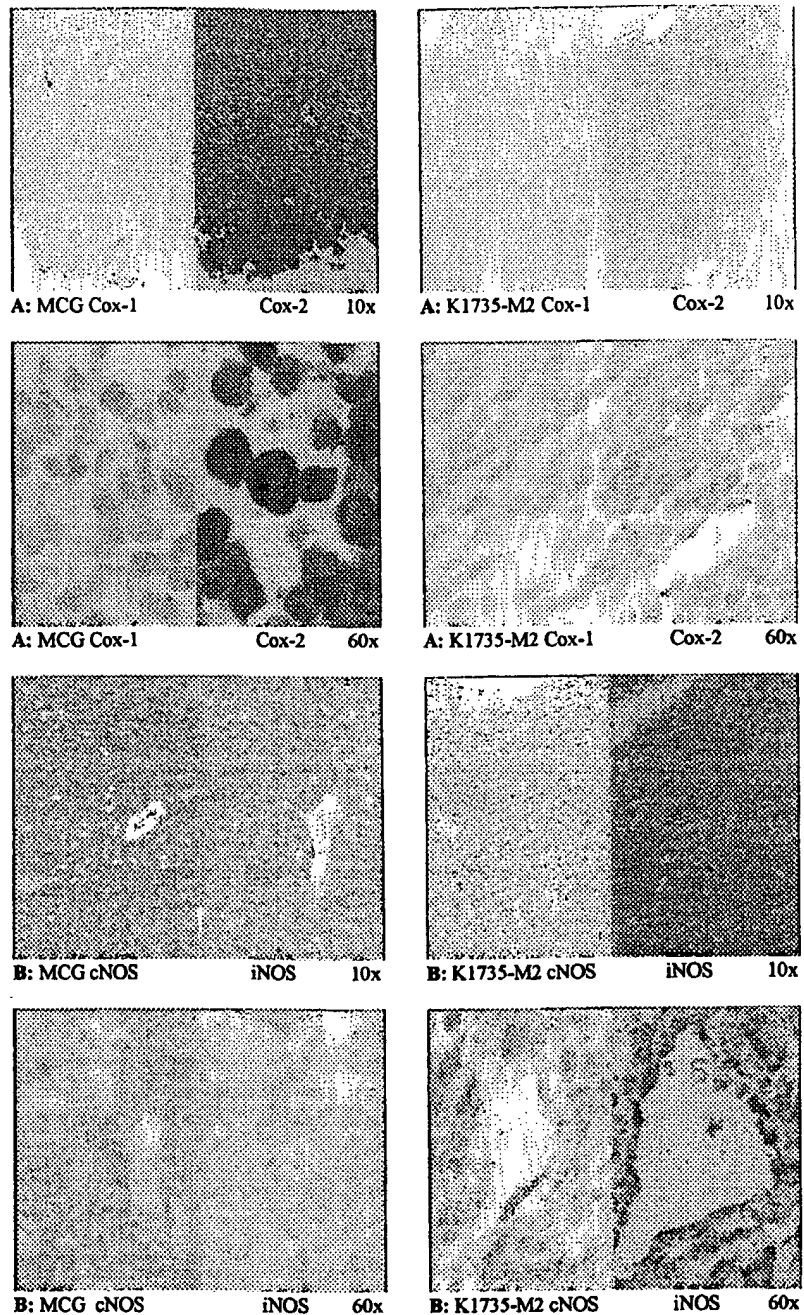


Fig. 4. A, immunohistochemical analysis of cell content of Cox-1 and Cox-2 protein (Cox-1 and Cox-2) in tumor tissue from MCG 101 tumors and melanoma (K1735-M2) at $\times 10$ and $\times 60$. B, cNOS and iNOS concentrations in the same tumor specimens.

was also associated with improved carcass fat-free dry weight, whereas the cumulative food intake was not significantly influenced (Table 1). Selective inhibitors (aminoguanidine and *S*-methylisothiourea) of iNOS had no beneficial effect on either tumor growth or nutritional state in any of the tumor models. The combination of Cox and NOS inhibition did not show any synergistic effect on tumor growth, food intake, or nutritional state in any of the tumor models. NOS inhibition (L-NOArg) had no effect on PGE₂ content in MCG 101 tumor tissue, whereas the host plasma PGE₂ concentrations decreased significantly from 3000 ± 600 pg/ml to 1521 ± 425 ($P < 0.05$). NOS inhibition by L-Name (1 mM) reduced only marginally proliferation of cultured MCG 101 ($6 \pm 2\%$; $P < 0.05$) and melanoma cells ($7 \pm 3\%$; $P < 0.05$).

Immunohistochemistry and Tumor Tissue mRNA. Cox-1 protein occurred at very low levels in both MCG 101 and melanoma

tumors, whereas Cox-2 protein was highly present in MCG tumors and almost not present in melanomas (Fig. 4A). Cox-2 was expressed 500-fold higher in MCG tissue compared with melanoma tissues evaluated by quantitative PCR (Fig. 5). Tumor tissue content of Cox-1 and Cox-2 protein was not changed by indomethacin treatment evaluated in MCG 101-bearing mice (not shown). Both tumors (MCG 101 and K1735-M2) contained substantial amounts of both cNOS and iNOS protein, with even distribution throughout the tumor tissue (Fig. 4B). Particularly, melanoma tumors seemed to have high levels of iNOS, which was confirmed by Western blot analysis (Fig. 6).

Provision of indomethacin to MCG 101-bearing mice increased tumor mRNA expression of cNOS but had no clear-cut effects on tumor expression of the transferrin receptor, angiogenin, VEGF, and bFGF (Table 2). Provision of L-NOArg to MCG 101-bearing mice also increased tumor cNOS mRNA. It also showed a trend to increased

COX-2

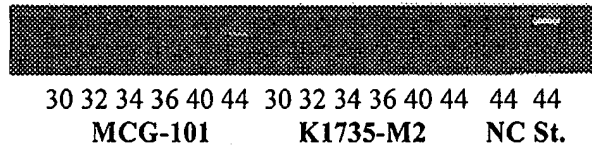


Fig. 5. Quantitative polymerase chain (reverse transcription-PCR) estimation of Cox-2 mRNA in tissue from MCG 101 and melanoma (K1735-M2) tumors as described in "Materials and Methods." Lanes (30–44) are numbers of PCR cycles. NC, negative control; St, gel electrophoresis standard DNA. Cox-2 expression was estimated to be >500-fold higher in MCG tumors compared with melanomas, accounting for β -actin expression (not shown).



Fig. 6. Western blot analysis of iNOS protein in tumor tissues (MCG-101 and K1735) from three different and randomly selected mice (1–3). The analyses were run on two different gels as described in "Materials and Methods." St, standard protein: Mouse Macrophage Lysate iNOS (Transduction Laboratories).

tumor expression of the transferrin receptor and VEGF, but L-NoArg provision was without effect on the expression of angiogenin and bFGF. The combination of indomethacin and L-NoArg had no synergistic effect on eNOS mRNA expression in tumor tissue (Table 2).

DISCUSSION

We have reported previously that indomethacin prolongs survival and improves food intake and the host nutritional state in both rats and mice bearing methylcholanthrene-induced tumors (5, 20). Similar effects have also been observed in cancer patients (6), although possible underlying mechanisms are not known. The indomethacin-responding MCG 101 tumor produces high amounts of PGE₂, whereas the malignant melanoma (K1735; Ref. 21), with low prostanoid production, did not respond to indomethacin treatment. We have also reported that indomethacin inhibition of PGE₂ production in MCG 101 tumors leads to decreased net proliferation and prolonged potential doubling time for tumor growth *in vivo*, whereas indomethacin had no major inhibitory effect on tumor cell DNA synthesis *in vitro* (1). Increased apoptosis, partly by depression of telomerase activity, may therefore be alternative mechanisms for retarded growth of tumors by Cox inhibition (33, 34). Also, the effect of indomethacin to decrease tumor growth *in vivo* was not dependent on intact immunity (20) and was not confined to any particular organ or vascular bed in the host, in accordance with reports that indomethacin attenuates

tumor angiogenesis (35). On the basis of our previous studies, we have concluded that prostaglandins in tumor tissue compartments promote local tumor growth by stimulation of tumor surrounding cells to produce growth factor(s) for tumor and matrix cell proliferation (1, 36). This broad interpretation has support by observations in more simplified cell models, where Cox activity is related to apoptosis, cell proliferation, cell differentiation, and regulatory peptides (37, 38). Additional information in the present study supports that beneficial effects by indomethacin *in vivo* may be a Cox-2-dependent phenomenon, because the specific Cox-2 inhibitor (L-745,337; Ref. 24) reduced tumor growth to the same extent as indomethacin (39).

A number of recent reports have indicated that NOS activities may either stimulate or retard tumor growth by effects that are superficially similar to mechanisms exerted by Cox pathways (10, 40). Such observations are interesting because NO mediates (14), or is at least related to, Cox activities and tumor growth (10, 41). Therefore, we decided to evaluate whether provision of NOS inhibitors at nontoxic doses attenuates cancer cachexia and tumor growth in addition to Cox inhibition (6, 14). For this purpose, we used two different tumor models: one model with cachexia being significantly dependent on Cox, and another model without cachexia, being unresponsive to Cox activity for tumor growth. Our results demonstrate that NOS inhibition by oral intake of arginine analogues (L-Name and L-NoArg) inhibited tumor growth *in vivo* by ~50% in both tumor models independently of eicosanoid formation, whereas cultured tumor cells were only marginally affected by NOS inhibition (<10%). These findings agree with conclusions on the role of NOS promotion for tumor growth in experimental and clinical cancer (21). However, specific inhibitors of iNOS activity had no effect in any of our tumor models, although iNOS protein was present in both kind of tumors. This phenomenon may indicate a poor correlation between the cellular content of iNOS and NO formation, as reported recently for arachidonic acid-metabolizing enzymes and generation of corresponding metabolites (39). The literature on NO formation and tumor growth is not unanimous (42, 43). Some studies report that low levels of NO promote tumor growth, whereas high levels may be inhibitory. Also, it is not clear whether stimulation of NO production by iNOS induction in tumor cells may promote tumor cell proliferation (44), whereas iNOS induction in surrounding host cells may prevent local tumor growth (45). Dong *et al.* (13) have reported that iNOS expression in K1735 melanoma cells was inversely related to metastatic growth and progression, findings which agreed with the observation that various clones of melanoma cells expressed iNOS above or below detection levels in tissues (46). Therefore, it is not possible to judge why our tumors, with detectable iNOS, did not respond significantly to iNOS inhibitors. Possible explanations may be insufficient drug concentrations after oral provision, or that iNOS is differentially important for local tumor growth and metastatic spread (39). With this uncertainty in mind, our findings suggest a model where downstream pathways for Cox and NO activation end up into a common effector mechanism(s) for MCG tumors, whereas other mechanisms may be present

Table 2 Northern blot analyses of the expression of the transferrin receptor (Tf-R), angiogenin, VEGF, cNOS, bFGF^a

	Area in %				
	Tf-R/ β -actin	Angiogenin/ β -actin	VEGF/ β -actin	cNOS/ β -actin	bFGF/ β -actin
TB controls	100	100	100	100	100
TB + Indo ^b	89	85	105	227 ^c	70
TB + NoArg	152 ^d	85	142 ^d	230 ^c	117
TB + Indo + NoArg	95	77	94	198 ^c	85

^a The drugs were provided in the drinking water for 10 days treatment as described in "Materials and Methods." There were five animals in each group.

^b Indo, indomethacin.

^c $P < 0.05$ versus TB controls with an estimated SD of $\pm 15\%$.

^d $P < 0.10$.

in K1735-M2 melanomas. These results both agree and disagree with models suggested by others for normal cellular physiology, where integrated functions between Cox and NOS production seem to be a general phenomenon (47–49).

Previous and present results suggest that the beneficial effects of indomethacin and NO inhibition may in part be host related. This conclusion is based on the observation that *in vivo* effects were more pronounced than *in vitro* effects, and that NOS inhibition in MCG-bearing mice did not decrease tumor content of prostaglandins but reduced circulating plasma PGE₂, which was associated with improvement in nutritional state (fat-free carcass weight). These observations suggest interaction between Cox and NOS pathways among host tissues and not only within tumor tissue (50–52). Alternatively, tumor growth inhibition by NO may not be independent of prostanoid pathways (53). However, if completely separate pathways occurred, one should expect additive effects by Cox and NOS inhibitors in MCG tumors, which was not observed.

We have earlier reported that provision of indomethacin to MCG 101-bearing mice increased tumor expression of various growth factors (IL-6, tumor necrosis factor- α , granulocyte-macrophage colony-stimulating factor, and transforming growth factor β_3) and decreased expression of bFGF and angiogenin, but it left the tumor expression of VEGF, epidermal growth factor, platelet-derived growth factor-A, platelet-derived growth factor-B, IL-1, and the transferrin receptor unchanged (1). The present study adds to previous information that transcriptional expression of cNOS mRNA in tumor tissue was increased 2-fold by either indomethacin or L-NOArg treatment (Table 2), confirmed by Western blot analyses.⁴ Thus, up-regulation of mRNAs in tumor tissue suggests that both Cox and NOS pathways are related to cellular growth factors in either tumor or in host (endothelial) cells. A decreased flow of prostanoid metabolites and of NO after provision of inhibitors up-regulated cNOS expression by negative feed back to support continuous tumor growth (54). These findings illustrate a phenomenon of cross-talk between Cox and NOS pathways (48). Although the model is not yet defined, our observations may include loops with positive and negative feedback for genes that may in the future be possible to use therapeutically. For example, cancer cells with mutated p53 had accelerated tumor growth associated with increased expression of VEGF for neovascularization related to hypoxia and subsequently assumed increased NO formation (11, 55, 56). It is generally anticipated that both indomethacin and NO influence the tumor vasculature, including effects on tumor blood flow, endothelial cells, and tumor nutrient uptake. However, unpublished work in our MCG 101-bearing mice has, thus far, not confirmed that Cox inhibition reduced angiogenesis in MCG 101 tumors. It now remains to be evaluated whether clinical tumors are sensitive to NOS inhibition in a way that may add to our treatment reported previously of cancer patients with Cox inhibitors alone (indomethacin), particularly in patients with tumors unresponsive to Cox intervention (6, 57).

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of tumor bearing mice with systemic inflammation and progressive anorexia-
cachexia.
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AU Cahlin, Christian; Gelin, Johan; Delbro, Dick; Lonnroth, Christina; Doi,
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EXPRESSION OF IL-1 β , IL-6, TNF- α AND COX-2 IN THE BRAIN OF TUMOR BEARING MICE WITH SYSTEMIC INFLAMMATION AND PROGRESSIVE ANOREXIA-CACHEXIA

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Cancer cachexia, with progressive wasting, anorexia and anemia in cancer is related to systemic inflammation. Thus, antiinflammatory treatment (indomethacin) prolongs survival and attenuates cachexia. It is however unclear how cytokines interact and explain the various parts of the cachectic syndrome although there are growing evidence that cytokine, expression in the brain may be important mediators of development of anorexia and cachexia in cancer disease. The aim of this study was to evaluate the expression of IL-1 β , IL-6, TNF- α and Cox-2 in relation to tumor growth and cachexia in a tumor model with systemic inflammation due to increased prostaglandin production (PGE₂).

Methods: A methylcholantrene induced sarcoma was implanted sc in C 57 bl mice. Pair fed and freely fed non tumor bearing C 57 bl mice served as controls. Body weight and food intake were registered daily. Net tumor growth was measured over 10 days treatment. Expression of IL-1 β , IL-6, TNF- α and Cox-2 in the central nervous system was determined by immunohistochemistry and subsequent computerized image analysis.

Results: Tumor bearing mice revealed reduced food intake and lost 10% body weight over 10 days due to rapid tumor growth. IL-1 β and IL-6 expression in ventromedial hypothalamic nuclei (VMH) were increased by 140% respectively ($p \leq 0.05$). IL-6 expression was increased by 130% ($p \leq 0.05$) in hippocampus whereas TNF- α expression in hippocampus was fourfold reduced ($p \leq 0.05$) in tumor bearing mice, compared to healthy controls. Cox-2 expression was three-fold increased in brain cortex, hippocampus and ventromedial hypothalamic nuclei (VMH) ($p \leq 0.05$) vs healthy controls.

Conclusion: Tumor growth was associated with reduced food intake and development of cachexia. This development of anorexia was associated with altered expression of cytokines in areas related to regulation of appetite in the central nervous system..

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INHIBITION OF HIV-1 REPLICATION AND ACTIVATION OF RNASE L BY THE METABOLICALLY STABLE, NON-TOXIC 2-SA DERIVATIVE, N⁶-BENZYL-3'-DEOXYADENYL-((2'-5')-3'-DEOXYADENYL-((2'-5')-3'-DEOXYADENYL-((2-2'')-N⁹-[(2''-HYDROXYETHOXY)METHYL]ADENINE (I).

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EXPRESSION OF IL-18, IL-6, TNF- α AND COX-2 IN THE BRAIN OF TUMOR BEARING MICE WITH SYSTEMIC INFLAMMATION AND PROGRESSIVE ANOREXIA-CACHEXIA

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Conclusion: Tumor growth was associated with reduced food intake and development of cachexia. This development of anorexia was associated with altered expression of cytokines in areas related to regulation of appetite in the central nervous system.

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Abstract Withdrawn

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INHIBITION OF HIV-1 REPLICATION AND ACTIVATION OF RNASE L BY THE METABOLICALLY STABLE, NON-TOXIC 2-5A DERIVATIVE, N⁶-BENZYL-3'-DEOXYADENYL- (2'-5')-3'-DEOXYADENYL- (2'-5')-3'-DEOXYADENYL- (2-2'')-N⁹-[(2''-HYDROXYETHOXY)METHYL]ADENINE (I).

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2-5A derivatives have been synthesized and characterized in these laboratories to act distal to the HIV-1-induced blockade in the 2-5A synthetase/RNase L antiviral pathway via activation of RNase L. The synthesis of I was accomplished by the phosphoramidite method. We have reported that I binds to and activates recombinant human RNase L, inhibits HIV-1 replication in SupT1 cells by inhibiting syncytia formation, HIV-1 RT and HIV-1 protein synthesis (manuscript in preparation). We have also reported that the RNase L inhibitor (RLI) is upregulated during HIV-1 infection and decreases RNase L activity. Therefore, the purpose of this study was to investigate the role of I in the activation of RNase L in HIV-1 infected H9 clones expressing different levels of RLI. This was accomplished by subcloning human RLI cDNA in the sense (VAS) and the antisense (VAS2) orientation in pcDNA3 followed by transfection into H9 cells. The empty vector was named VV. The 2-5A binding and activity of RNase L were measured by RNA cleavage and 2',5'-[³²P]AA-8-azidoA photolabeling assays. We observed that I binds to and activates RNase L in uninfected H9 cells. RNase L activation was dependent on RLI levels. The over-expression of RLI caused a decrease in RNase L activation by I concomitant with an increase in HIV-1 replication as determined by an increase in HIV-1 proteins. In contrast, reduction of RLI levels reverse the inhibition of RNase L activation and led to a decrease in HIV-1 replication. This work was supported in part by United States Public Health Service grant R01 AI 34765 (to RJS).

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ROLE OF RECOMBINANT OVINE IFN- τ ON OXYTOCIN (OT)-STIMULATED UTERINE RECEPTIVITY DURING OESTROUS CYCLE AND PREGNANCY

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In ruminants, IFN- τ is a powerful embryonic signal of pregnancy. It maintains luteal progesterone secretion by inhibiting PGF-2 α pulsatile secretion which leads to luteolysis during oestrous cycle. Moreover it prevents OT-receptor expression which is directly implicated in the luteolytic loop: luteal OT-uterine PGF-2 α . In the present study, we investigated whether IFN- τ is able to block OT-stimulated uterine receptivity as the conceptus does during the establishment of pregnancy. roIFN- τ treated ewes were compared to both pregnant (positive control) and cyclic ewes (negative control). On Day 9 post oestrous, cyclic animals (n=18) were treated by uterine route with a single dose of roIFN- τ (1.7 mg, 10⁸ IU/mg) covalently bound to carboxymethyl biogel. Luteal function was controlled by progesterone measurements. From Days 13 to 16, pregnant ewes (n=4), cyclic ewes (n=3) and roIFN- τ treated cyclic ewes (n=6) received successively intravenous bolus injections of NaCl (0.9%), 1 IU oxytocin 1h later and 5 IU 4.30 h later. The OT-induced PGFM (13-14 dihydro, 15-keto PGF-2 α) release was estimated by radioimmunoassay. To determine whether OT-induced PGFM response ability was closely related to OT receptor concentrations, pregnant ewes (n=12), cyclic ewes (n=12) and roIFN- τ -treated cyclic ewes (n=12) were slaughtered on Days 12, 14 and 16 (4 animals per stage). In pregnant group, no significant OT-induced PGFM response was observed in all treatments and stages. In cyclic group, plasma PGFM concentrations increased from D14 with a maximum on D16 whatever OT dose used. In roIFN- τ -treated cyclic group OT-induced PGFM response slightly increased from D14 to D16 when the higher dose (5 IU) of OT was used. This increase was significantly higher than that observed in pregnant ewes ($p < 0.05$) and much lower than that of cyclic ewes ($p < 0.05$) on Day 16. Endometrial OT receptor concentration remained in basal level from D12 to D16 in pregnant ewes but largely increased on D14 and D16 in cyclic ewes ($p < 0.05$ and $p < 0.01$, respectively). In roIFN- τ -treated cyclic ewes, OT receptor concentrations slightly increased on D16 ($p < 0.05$). These results confirm the key rôle of IFN- τ on inhibition of OT-induced PGFM response and endometrial OT receptor expression which leads on maintenance of CL function. However, it seems that the effect of IFN- τ alone is less efficient than that produced from the conceptus, thus suggesting that other embryonic and/or maternal factors may be involved in the mechanism of luteolysis control.